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(71) Applicant (for all designated States except US): DADE BEHRING MARBURG GMBH [DE/DE]; P.O. Box 11 49, D-35001 Marburg (DE).		
(72) Inventors; and		
(75) Inventors/Applicants (for US only): DELANGHE, Joris [BE/BE]; Brusselsesteenweg 105, B-9300 Aalst (BE). LANGLOIS, Michel [BE/BE]; Oudenaardsesteenweg 6, B-9000 Gent (BE). DE BUYZERE, Marc [BE/BE]; Sint-Pietersgroenestraat 129, B-8000 Brugge (BE).		
(74) Agents: VAN REET, Joseph et al.; Gevers Patents, Hollandstraat 5, B-1831 Diegem (BE).		

**(54) Title:** METHOD AND KIT FOR DETERMINING THE PHENOTYPE OF A HAPTOGLOBIN AND USE THEREOF**(57) Abstract**

The method for determining the phenotype of a haptoglobin in a biological fluid, characterized in that it comprises the steps of (a) contacting the biological fluid with a haptoglobin binding partner, in particular with *Streptococcus pyogenes*, which has at least two locations by which it can bind to haptoglobin so as to be able to agglutinate haptoglobins and/or to be agglutinated thereby to different degrees, depending on the haptoglobin phenotype; (b) measuring the degree of agglutination; and (c) determining, based on the degree of agglutination, the phenotype of the haptoglobin in the biological fluid. The kit comprises the haptoglobin binding partner and optionally a reference haptoglobin sample. This kit and method can be used for estimating a patient's prognosis following viral infection, for determining graft survival following liver transplantation, and for improving the interpretation of different laboratory parameters. Compared to existing assays, they enable a quick determination of the haptoglobin phenotype.

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**"Method and kit for determining the phenotype of a haptoglobin and use thereof"**

This invention relates to a method and a kit for determining the phenotype of a haptoglobin, in particular a human haptoglobin, in a biological fluid.

Human haptoglobins (Hp) have been well studied; they were discovered over 40 years ago and they fulfil many biological key roles such as plasma transport of haemoglobin and interactions with the immune system. All haptoglobins contain two classes of polypeptide chains, beta chains and alpha chains. Beta chains are identical in all haptoglobins, whereas the alpha chains show two forms giving rise to three Hp phenotypes: Hp 1-1, Hp 2-1, Hp 2-2.

Many functional differences exist between the Hp phenotypes, which appear to have important clinical consequences (Langlois & Delanghe, Clin Chem 1996;42:1589-1600). The Hp 2-2 type is characterized by a lower haemoglobin-binding capacity and hence a less efficient protection against oxidative stress, a higher degree of nitric oxide (NO) inhibition, and a less pronounced inhibition of prostaglandin synthesis (Langlois & Delanghe, Clin Chem 1996;42:1589). Furthermore, haptoglobin has angiogenic effects (Hp 2-2 being more angiogenic) (Cid MC et al., J Clin Invest 1993;91:977), agglutinates streptococci by binding to the T4 antigen (Nature 1978;271:373), and is involved in homing and trafficking of leukocytes (Hp binds to the leukocyte adhesion molecules CD11b/CD18 and CD22) (J Biol Chem 1995;270:7543, J Immunol 1996;156:2542). The fact that haptoglobins bind to T4 antigens

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has already been used for developing an ELISA test for quantitation of human haptoglobin (Katnic I. et al, Arch. Immunol. Ther. Exp. Warsz 1993;42:105-9). This test however does not enable to make a distinction between the different haptoglobin phenotypes since the binding site of the T4 antigen on haptoglobin is the same for the different haptoglobins.

The above described functional properties have important clinical consequences. The Hp 2-2 type is an independent risk factor in refractory essential hypertension and is a predictive marker for target organ damage in essential hypertension (J Hypertens 1993;11:861, J Cardiovasc Risk 1995;2:131) . Also in acute myocardial infarction (N Engl J Med 1982;307:457 ) and in coronary atherosclerosis (Atherosclerosis 1997;132:215), the Hp 2-2 phenotype was found to be an important independent risk factor. After correction of the more classical cardiovascular risk factors (e.g. smoking, cholesterol, hypertension,...), the cardiologist remains unable to predict which patient will accumulate atherosclerotic lesions more rapidly. Therefore, knowledge of a genetic predisposition (e.g. Hp type) leads to a better prevention and a more careful follow-up of the cardiovascular patient.

The Hp 2-2 type is associated with a higher mortality of HIV-infection (Trop Med Int Health 1997;11:1102). Kaplan-Meier survival analysis showed a median survival time of approximately 7 years for Hp 2-2 patients versus 11 years for patients carrying another Hp type. This has been explained by a less efficient protection against iron-driven oxidative stress, resulting in a higher viral replication rate. This enhanced viral replication increases the risk for developing therapy-resistant HIV strains, as can be observed in patients with therapeutic resistance towards the potent protease inhibitors. Therefore, knowledge of the patients' Hp type predicts the prognosis of HIV infection and leads to better tailored therapeutic strategies.

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Another clinical application can be found in the management of the patient following liver transplantation. Since haptoglobin is exclusively produced by the liver tissue, liver transplantation is a biologically unique condition in which the Hp type of a patient changes (if different from the pre-transplant Hp type). The patients' Hp type in serum exclusively will depend on the haptoglobin phenotype produced by the liver graft.

In a series of 177 patients who underwent a liver transplantation, it became clear that for transplantations with a primary cause of viral hepatitis ( $n = 88$ ), survival of patients who have received a graft expressing the Hp 2-2 phenotype were characterized by a poorer prognosis ( $P < 0.03$ ) as compared with liver transplanted patients who have received a Hp 2-1 or an Hp 1-1 producing graft. As the time frame for controlling the quality of the graft prior to transplantation is very short, a fast Hp phenotyping method could therefore contribute to a better outcome in liver transplantation.

A rapid Hp typing method is useful for paternity testing in forensic medicine. Furthermore, the Hp polymorphism has a genetic influence on the reference values of a broad range of laboratory parameters, not only on the serum Hp concentration (lower for the Hp 2-2 phenotype) but also on plasma lipids (total and LDL-cholesterol), plasma proteins (ferritin, immunoglobulin A, ceruloplasmin), and other biochemical parameters (serum iron, transferrin saturation, vitamin C, vitamin E) (Langlois & Delanghe, Clin Chem 1996;42:1589, Langlois et al, Clin Chem 1996;42:1722). For instance, slight hemolysis in a Hp 2-2 patient can often be confused with a congenital Hp deficiency.

Also in hematology, reference values for peripheral blood B-lymphocytes and CD4+ T-lymphocytes are dependent on Hp type (Langlois et al; Eur J Clin Chem Clin Biochem 1997;35:199). This may

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lead to an over- or underestimation of CD4+ cell counts when using the fixed CDC classification in the diagnosis of AIDS. Therefore, additional determination of Hp type using a rapid and simple test is helpful to avoid some problems in laboratory medicine.

In summary, the determination of Hp type is an interesting additional test for the clinicians' diagnosis and in the prophylactic and therapeutic management of the patient.

Up to now, haptoglobin phenotyping in serum or plasma is usually performed by starch gel electrophoresis (O. Smithies ; Biochemical Journal 1955, 61: 629-41). A drawback of this known technique is that it is slow, laborious and requires the use of electrophoresis equipment.

An object of the present invention is to provide a new method for phenotyping haptoglobins in a biological fluid which is much faster and easier to perform.

To this end, the method according to the invention comprises the steps of :

- a) contacting the biological fluid with a haptoglobin binding partner which has at least two locations by which it can bind to haptoglobin so as to be able to agglutinate haptoglobins and/or to be agglutinated thereby to different degrees, depending on the haptoglobin phenotype;
- b) measuring the degree of agglutination ; and
- c) based on the degree of agglutination, the phenotype of the haptoglobin in the biological fluid.

The degree of agglutination can be determined visually or by means of conventional techniques such as turbidimetry and/or nephelometry. In view of the higher accuracy of nephelometry, a particular preference is given to this latter technique although other techniques may also be suitable.

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As biological fluid, a sample of all kinds of different biological fluids can be taken. Examples of such biological fluids are blood, plasma, serum, liquor, urine, cell extracts or tissue extracts.

The haptoglobin binding partner which is used in the method according to the invention may be of different types and dimensions. Essential for the binding partner is that it has at least two locations by which it can bind to haptoglobin so as to be able to agglutinate haptoglobins and/or to be agglutinated thereby and this to different degrees, depending on the haptoglobin phenotype.

The locations by which said binding partner can bind to haptoglobins may be formed by a peptide, an antibody, an  $F_{ab}$  or  $F_{ab'}$  or  $F(ab')_2$  fragment of an antibody, a lectin, a cell receptor, a molecular imprint of a haptoglobin, a bacterial antigen, and/or fragments thereof containing the respective haptoglobin binding site. As bacterial antigen, especially the T4 antigen of Streptococcus pyogenes appeared to be very suited.

The different degree of agglutination can be based on the specificity of the haptoglobin binding location itself. A specific binding location may comprise for example the  $F_a$ ,  $F_{ab'}$  or  $F(ab')_2$  fragment, or even a smaller portion containing the antigen binding site, of an antibody which specifically binds the  $\alpha 2$  chain of a haptoglobin of phenotype Hp 2-1 or HP 2-2 but not an  $\alpha 1$  chain. The  $\alpha 2$  chain is the result of a mutation based on an unequal crossing-over. It comprises 142 amino acids whilst the  $\alpha 1$  chain comprises only 83 amino acids. Immunologically, both chains comprise mainly the same epitopes, only in the crossing-over region, the  $\alpha 2$  chain has a unique sequence of amino acids. This unique sequence comprises the following sequence of amino acids : ala val gly asp lys leu pro glu cys glu ala asp asp gly gln pro pro pro lys cys ile. Suitable epitopes for making selective antibodies can thus

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be selected within this sequence. In order to be selective, the epitopes have to comprise at least the portion "glu ala asp" from the previous sequence.

For making specific antibodies, synthetic peptides corresponding to the selected epitopes can first of all be made. Such synthetic peptides capable of raising haptoglobin-specific antibodies have to be coupled to an immunogenic protein (carrier protein) e.g. keyhole limpet hemocyanin, bovine serum albumin and the like. Lines of somatic cells immunised against the synthetic peptides can be obtained by immunisation of suited animals e.g. mice (e.g. BALB/c), rabbits, sheeps, goats, etc. The preparation of monoclonal antibodies could be done e.g. using the method of Fazekas, de St. Groth S. and Scheidegger, D., "Production of monoclonal antibodies: strategy and tactics", J. Immunol. Meth., 35, 1, 1980.

In general, the host (mice, rabbits, sheeps, goats, etc.) is immunised by administering the peptide-protein conjugate using any suitable injection method, either intraperitoneally, intravenously, subcutaneously, etc. Suitable adjuvants (e.g. Freund's) may be included in the immunisation protocol.

The initial immunisation with the antigen is normally followed by several booster injections given periodically at intervals of several weeks. In case of monoclonals the immunised somatic cells, preferably spleen cells, must then be fused with a myeloma cell line (e.g. SP2/0) to produce hybridomas capable of secreting specific antibodies.

The detection of specific antibodies (hybridomas or immune serum) may be performed by any suitable assay such as enzyme immunoassays, radioimmunoassays and/or nephelometric or turbidimetric assays. Appropriate screening procedures comprise the selection of specific antibodies on their binding abilities to the synthetic

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peptides, to the peptide-protein conjugates, to the carrier proteins and to the different haptoglobins phenotypes. Those antibodies are preferred which bind specifically to the synthetic peptides, to the peptide portion of the peptide protein conjugates and to haptoglobin Hp 2-2 and/or 2-1.

Maintenance of the hybridomas is accomplished by the use of appropriate standard tissue culture media containing preferably foetal calf serum.

Production of higher yields of monoclonals could be conducted via ascites or serum free mass cell culture (roller bottles, fermenter, etc.).

How to isolate the desired antibodies (from culture supernatant, ascites or serum) is a matter of routine art. Well known techniques are salt precipitation, gel chromatography, ion exchange chromatography, affinity chromatography (e.g. Protein A) and the like.

Antibodies which are specific to the  $\alpha_2$  chains can be used as such as haptoglobin binding partners for agglutinating haptoglobins of phenotype Hp 2-2 and 2-1. Due to the fact that the Hp 2-2 phenotype comprises only  $\alpha_2$  chains whilst the Hp 2-1 phenotype comprises  $\alpha_1$  and  $\alpha_2$  chains, a difference in agglutination degree can be observed.

In the above described embodiment, the binding partner is formed by a molecule, namely by an antibody, which is able to form a lattice or network with the haptoglobin molecules and which is thus capable of agglutinating them. Instead of natural molecules, it is also possible to develop artificial molecules which are able to agglutinate haptoglobins. This can be done by directly (chemically) or indirectly (e.g. using biotin and avidin or streptavidin) coupling for example haptoglobin binding molecules such as antibodies, antibody fragments, eucaryotic and/or prokaryotic cellular receptors or parts thereof to other molecules, possibly in combination with other haptoglobin binding molecules which

are specific or non-specific for the different haptoglobin phenotypes, for example a T4 antigen or the binding portion thereof.

In a variant embodiment, the antibodies, in particular polyclonal or monoclonal antibodies, or fragments thereof containing the haptoglobin binding sites, such as  $F_{ab}$ ,  $F_{ab'}$  or  $F(ab')_2$  fractions or even smaller fractions, can be adhered to carrier particles, in particular to synthetic or mineral particles but also to natural cells or cell fragments. In this way, the sensitivity of the assay can be improved. The particles have such dimensions that the network formed thereby, or in other words the degree of agglutination, can easily be determined e.g. turbidimetrically and/or nephelometrically. The dimensions of the carrier particles are preferably comprised between  $0.02\mu$  and  $2\mu$ , and most preferably between  $0.1\mu$  and  $0.8\mu$ , in view of improving the sensitivity of the assay.

Among the carriers which can be employed are latex particles for example based on polystyrene, polymethacrylate, polyacrylate, polyvinylacetate, polyvinylpyridine, vinylchlorid-acrylate, polybutadienestyrene or polybutadieneacrylonitrilstyrene copolymers. Useful might be as well phenolic resins or finely divided cellulose or amino cellulose particles. The carriers used can be further erythrocytes, the substances bentonite, cholesterol crystals, quartz or micro particles from inorganic oxides, like silicium dioxide, alumina oxide or other finely dispersed minerals. Disperses metals as for instance gold, silver or others might be used as well. Useful might be further dispersed bacteria as staphylococcus or streptococcus, bacillus prodigios, rickethsia or cell membrane fragments.

The binding of antigens or antibodies to the carriers could be accomplished by adsorption or covalent techniques.

Examples for a covalent binding are

- a) the aldehyde method given in the US patent 4,448,908;

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- b) the carbodimide method according to Gross et al.: Immunochemistry, Vol. 5, p. 55, 1968;
- c) the acid chloride method and acid anhydride method according to Erlanger et al.: Journal of Biological Chemistry Vol. 228, p. 713, 1957;
- d) the isocyanate method - Goodfriend et al.: Canadian Journal of Biochemistry and Physiology, Vol. 36, p. 1171, 1958.

According to the invention, it has been found that, in order to obtain a measurable difference in degree of agglutination between the different haptoglobin phenotypes, the locations on the binding partners by which the haptoglobins are bound do not have to be specific for the different haptoglobin phenotypes. It is presumed that the different degrees of agglutination can be attributed to the different molecular weights of the haptoglobin phenotypes, in other words to their different sizes, and to the different contents of  $\alpha_1$ ,  $\alpha_2$  and  $\beta$  chains. The average molecular weight decreases more particularly from the Hp 2-2 phenotype, over the Hp 2-1 phenotype to the Hp 1-1 phenotype.

Tests have been carried out with bacterial strains, in particular with *Streptococcus pyogenes*, which carry the T4 antigen. This antigen binds to the  $\beta$  chain of haptoglobins. Although the binding itself is not specific, the degree of agglutination is sufficiently different to make a distinction between the different haptoglobin phenotypes, in particular also between the Hp 2-1 and Hp 2-2 phenotypes. In this respect, it should be noted that the fact that *Streptococcus pyogenes* carrying T4 antigens can be agglutinated by Hp 2-2 and Hp 2-1 but not by Hp 1-1 was already disclosed in Nature 1978; 271:373. However from this publication it is not clear that, based on this property, an agglutination assay can be developed for making a distinction between the different haptoglobin phenotypes, especially not for making a distinction between the Hp 2-1 and Hp 2-2 phenotypes.

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Instead of using entire bacteria, one could also consider using fragments thereof carrying the bacterial antigen which forms the haptoglobin binding location. Use could be made for example of a bacterial lysate.

Beside procaryotic bacterial cells, also some eucaryotic cells or fractions thereof showing haptoglobin receptors may be suited for obtaining the desired agglutination reaction. Suitable receptors are in particular CD receptors, more particularly CD22 receptors. These receptors may be located on animal or human cells, in particular on leukocytes.

According to the invention, the haptoglobin binding locations do not necessarily have to be situated naturally on the carrier particles but, as explained hereinabove in relation to the specific antibodies, it is possible to attach the haptoglobin binding fragments by adsorption and/or covalent binding to other carriers, in particular to synthetic or mineral carrier particles or to natural cells or fragments thereof. In case the binding fragments are not specific for different haptoglobin phenotypes, the carrier particles have preferably a diameter of between 0.5  $\mu$  and 4  $\mu$ , and more preferably of between 1  $\mu$  and 3  $\mu$ . These diameters are indeed not only important for the determination of the degree of agglutination by turbidimetry or nephelometry, but it has now been found that the dimensions of the carrier particles play also a role in the different agglutinating properties of the haptoglobin phenotypes. The dimensions of the carrier particles are more particularly chosen such that the relatively small haptoglobin molecules of the Hp 1-1 phenotype are not or nearly not able to agglutinate the carrier particles whilst the larger molecules of the Hp 2-1 phenotype and the on average still larger molecules of the Hp 2-2 phenotype are able to agglutinate them and this more particularly even to a different degree for the Hp 2-1

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and HP 2-2 phenotypes. In a particular embodiment, the carrier particles may additionally be adhered to one another, in particular to produce chains of 5 to 20 particles, similar to the chains formed by *Streptococcus pyogenes*.

For providing the locations to which the haptoglobins can bind on the carrier particles of the haptoglobin binding partner, use can not only be made of the above described specific antibodies or antibody fragments and the T4 antigen, but further of other substances which bind to haptoglobins. As mentioned already hereinabove, these substances may generally include a peptide, an antibody, an  $F_{ab}$ ,  $F_{ab'}$  or  $F(ab')_2$  fragment of an antibody, a lectin, a cell receptor, a molecular imprint of a haptoglobin, a bacterial antigen, and/or fragments thereof containing the respective haptoglobin binding site. Use can in particular be made of poly- or monoclonal antibodies against haptoglobins which do not have to bind specifically to one of the haptoglobin phenotypes. Moreover, one could consider using for example the leukocyte adhesion molecules (lectins) CD11b/CD18 and/or CD22 as haptoglobin binding sites. At least two binding locations are provided on each carrier particle so that a network or lattice can be formed in the biological fluid, resulting in the intended agglutination.

The present invention still provides a further solution for obtaining the required difference in agglutination degrees. Instead of using carrier particles of a particular size, the haptoglobin binding locations can indeed also be provided on molecules, in particular macromolecules, which are thus able to agglutinate haptoglobins of a different size to a different degree due to the steric hindrances. The degree of agglutination can then be measured after a time sufficient to obtain the final agglutination or sooner so that also the binding kinetics play a role in the measured degree of agglutination.

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The degree of agglutination is not only dependent on the haptoglobin phenotype, but also on the concentration of the haptoglobin in the biological fluid. In a preferred embodiment, the concentration of the haptoglobin is therefore also measured and taken into account when determining the phenotype on the basis of the degree of agglutination. Preferably, the relationship between the concentration of a reference haptoglobin in the biological fluid and the degree of agglutination is first determined for at least one reference haptoglobin phenotype, in particular for a haptoglobin phenotype which causes a sufficient degree of agglutination. Normally, this will be the Hp 2-2 or Hp 2-1 phenotype, preference being given to either both of them or to the Hp 2-2 phenotype. The phenotype of the haptoglobin in the biological fluid is then determined by comparing the relationship between the measured concentration of the haptoglobin and the measured degree of agglutination with the same relationship which has first been established for the reference haptoglobin. It has indeed been found that there is a significant difference in agglutination properties, for a same haptoglobin concentration, between the haptoglobins of phenotypes Hp 1-1, Hp 2-1 and Hp 2-2.

In a particularly preferred embodiment of the method according to the present invention, for determining the phenotype of a haptoglobin in a biological fluid, the measured degree of agglutination is correlated by means of the relationship, which has been established between the concentration and degree of agglutination for the reference haptoglobin, to a particular concentration of the reference haptoglobin, namely to that concentration of the reference haptoglobin which provides the same degree of agglutination. Then, the ratio between this concentration and the actually measured concentration is calculated. The thus calculated ratio is indicative of the phenotype of the

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haptoglobin in the biological fluid. Indeed, when haptoglobin Hp 2-2 has been taken as reference haptoglobin, a calculated value near 1 means of course that the haptoglobin is of the Hp 2-2 phenotype. Experimentally, it has been found that this ratio is further situated near 0.5 in case the haptoglobin is of the Hp 2-1 phenotype and even much smaller, i.e. near 0.125 in case the haptoglobin is of the Hp 1-1 phenotype.

In a particular embodiment of the kit according to the present invention for determining the phenotype of a haptoglobin in a biological fluid is provided for establishing the relationship between the degree of agglutination and the haptoglobin concentration for at least one reference haptoglobin phenotype. Indeed, it comprises, in addition to the above described haptoglobin binding partner, at least one sample containing an haptoglobin of a predetermined phenotype. This sample may be a sample of the same biological fluid, which may for example have been lyophilised or otherwise treated to increase the shelf life thereof, or may be an artificially prepared solution. Moreover, the haptoglobin sample may be a sample of a solid haptoglobin or a substantially pure preparation thereof. The kit may additionally comprise a solution for diluting or dissolving the haptoglobin sample.

By means of this sample the degree of agglutination can be determined for one or more concentrations (dilutions) of the predetermined haptoglobin. From practical tests performed with suspensions of *Streptococcus pyogenes* as haptoglobin binding partner, it appeared to be important to be able to determine, when performing a test or a series of tests, each time the degree of agglutination for one or more concentrations of the predetermined haptoglobin, or in other words to calibrate the measurement of the degree of agglutination.

The haptoglobin calibration or control sample included in the kit contains preferably a haptoglobin of the Hp 2-2 or Hp 2-1

phenotype, and most preferably a haptoglobin of the Hp 2-2 phenotype, since such phenotype generally causes the highest degree of agglutination.

As mentioned hereinabove, different types of haptoglobin binding partners may be appropriate for obtaining the desired agglutination reaction. First of all, use can be made of bacteria, in particular of *Streptococcus pyogenes*. In order to increase the shelf life thereof, these bacteria, or fragments thereof, can also be included in the kit in lyophilised form. In view of the shelf life and also in view of obtaining more easily a standardised agglutination reaction, preference may be given to a non-living haptoglobin binding partner. The binding partner may comprise for example carrier particles having the locations to which the haptoglobins can bind artificially attached thereto. The haptoglobin binding partner may in particular comprise an antibody which specifically binds an  $\alpha_2$  chain of a haptoglobin of phenotype Hp 2-1 or Hp 2-2 but not an  $\alpha_1$  chain or at least the binding site of said specific antibody. As described hereinabove, this antibody is preferably attached to carrier particles. When the agglutination reaction, i.e. the haptoglobin binding partner, can be sufficiently standardised, the phenotyping can be carried out without requiring the use of a sample containing a reference haptoglobin, or in other words the kit for determining the phenotype of a haptoglobin may essentially consist of the haptoglobin binding partner itself.

In a particular embodiment of the kit according to the invention, it further comprises a preparation of antibodies which are immunoreactive with all the different haptoglobin phenotypes to a same degree. Such preparation enables to determine the haptoglobin concentration in the biological fluid, and if still necessary, in the haptoglobin calibration sample, by immunonephelometry.

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According to the invention, the above described method and kit for determining the haptoglobin phenotype can be used for different purposes.

First of all, the use of the method or kit according to the invention is proposed for assessing atherosclerotic risk. In case a Hp 2-2 phenotype is found, this is correlated with a higher risk.

The use of the method or kit according to the present invention is further proposed for estimating a patient's prognosis following viral infection, in particular a HIV infection. In this particular use, the determination of a Hp 2-2 haptoglobin phenotype is correlated with a worsened prognosis.

The use of the method or kit according to the invention is moreover proposed for determining graft survival following transplantation, in particular liver transplantation, the determination of a Hp 2-2 haptoglobin phenotype of the transplanted liver being in this case correlated with a lower survival.

The use of the method or kit according to the present invention is finally also proposed for improving the interpretation of laboratory parameters such as serum haptoglobin concentration, plasma lipids, in particular total lipids and LDL-cholesterol, plasma proteins, in particular ferritin, immunoglobulin A and ceruloplasmin, and other biochemical parameters such as serum iron, transferrin saturation, vitamin C and vitamin E. Reference values established for these parameters are indeed also to be correlated with the haptoglobin phenotype.

Further particularities and advantages of the invention will become apparent from the following example of a method according to the invention. This example is, however, not intended to limit the scope

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of the invention. The results obtained are indicated in the drawings wherein :

Figure 1 shows a typical calibration curve of the agglutination assay performed in Example 1 ; and

Figure 2 shows the relationship between the relative agglutination and the haptoglobin concentration and this for the different haptoglobin phenotypes.

#### Example

In this example, the phenotype of the haptoglobin present in different blood serum samples was determined. Use was made as binding partner for the agglutination reaction of bacterial cells, more particularly of freshly grown *Streptococcus pyogenes* strains carrying the T4 antigen.

#### Preparation of the bacterial suspension.

The *Streptococcus pyogenes* strains had been grown overnight on commercial blood agar media plates (Becton Dickinson, Erembodegem, Belgium) at 37 °C prior to diagnostic use. The colonies formed on the plates were transferred into a 0.9% NaCl solution. This solution was agitated to obtain a homogeneous bacterial suspension. The viscosity of the bacterial suspension was increased by final concentrations of 10% glycerol so that a stable suspension was obtained. The suspensions were diluted or additional bacteria were added until the absorbance (at a wavelength of 600 nm) in a standard 1 cm-cuvette was equal to about 0.250. The bottle containing the suspension was well agitated before use, at least when there were longer periods between the measurements.

#### Measurement of the haptoglobin concentrations.

Haptoglobin concentrations were measured immunonephelometrically using the Behring nephelometer II (BN II) of

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Behring Diagnostics GmbH, D-35001 Marburg, Germany. The nephelometer was calibrated against the international CRM 470 protein standard (reference: Whicher et al. Clin Chem 1994;40:934-8). The agglutination reaction was obtained by means of the rabbit anti-human haptoglobin antibodies provided in the quantitative haptoglobin determination kit of Behring Diagnostics. Further, the procedure provided in this kit was followed. More information about this procedure can be found in Fink PC et al. J. Clin. Chem. Clin. Biochem. 1989;27:261-76.

Measurement of the degree of agglutination.

The degrees of agglutination in the different serum samples were also measured by means of the BN II nephelometer. For obtaining the required agglutination, 50 µl of the above bacterial suspension was each time added to 20 µl serum sample.

Calibration of the assay was obtained using a serum pool originating from a healthy blood donor carrying the Hp 2-2 phenotype which was chosen in this example as reference haptoglobin. Haptoglobin concentration of this serum pool was determined as described hereabove and was adjusted by means of a 0.9 % NaCl solution to a concentration of 1 g/l Hp 2-2. This standard was arbitrarily set at 100 agglutination units/g Hp 2-2. Serial dilutions of the serum pool in physiological (0.9 % sodium chloride) saline solution (100%, 40%, 20% of the initial concentration) were made to construct the calibration curve. For constructing this curve, the following program was set on the BN nephelometer :

Test No.	53	Abbreviation	HTYP
Test name	HAPTOCOC		
Sample volume (µl)	20	Sample dil. 1 :1.0	
		Minimal-dilution 1.0	
Reagent 1 vol. (µl)	50	T4	

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Reagent 2 vol. (µl)	0	
React. buffer vol(1)	50	N Reaction Buffer
React. Buffer vol(2)	50	
Measuring time (min)	12	Fixed Time
Standard		pool
No.of Std. points	4	
First dilution	1 : 1.0	
Deviation allowed (%)	99.9	
Validity (days)	7	
Conc. unit		units
Measuring range (units)		
Lower level	0.00	
Upper level	1300.0	

For each of the four concentration, corresponding to 1.0 ; 0.4 ; 0.2 and 0.1 g Hp 2-2/l, the difference in value measured before and after the addition of the bacterial suspension was calculated and was used to indicate the degree of agglutination. These delta measurement values were respectively 1799 ; 584 ; 195 and 74 and are shown in Figure 1. In this Figure, both the agglutination units and the corresponding Hp 2-2 concentrations have been indicated on the X-axis.

With the same nephelometer and the same bacterial suspension, the difference in measured value was again determined for a number of unknown serum samples. The delta measurement values corresponded, according to the graph in Figure 1, to a certain amount of agglutination units or to a particular concentration of Hp 2-2 haptoglobin, namely to a concentration of Hp 2-2 haptoglobin giving the same degree of agglutination.

Determination of the haptoglobin phenotype.

Before determining the phenotype of a haptoglobin of an unknown phenotype, the degree of agglutination was first of all measured for a large number of serum samples containing the different haptoglobin phenotypes. For each of these samples, the degree of agglutination and the haptoglobin concentration was measured as set forth hereabove. Further, based on the calibration graph shown in Figure 1, or similar calibration graphs established when performing the different measurements, the measured degree of agglutination was correlated to the corresponding Hp 2-2 concentration or, in other words, to the amount of agglutination units (relative agglutination in %). A linear regression analysis was applied to the obtained data about the measured concentrations and the relative agglutination percentages and the calculated regression lines for the different haptoglobin phenotypes were shown in Figure 2. When both on the X and the Y axis, the measured haptoglobin concentration and the relative agglutination is expressed in g/l, the regression coefficients were respectively about 1 for the HP 2-2 phenotype, 0.5 for the Hp 1-1 phenotype and 0.125 for the Hp 1-1 phenotype.

For determining the phenotype of the haptoglobin in unknown serum samples, the haptoglobin concentration of these samples is measured in addition to the degree of agglutination and the corresponding concentration of the reference haptoglobin Hp 2-2. These measurements of the haptoglobin concentration were also done by means of the BN II nephelometer according to the procedure referred to hereabove. Subsequently, the ratio's between the corresponding Hp 2-2 concentrations, determined on the basis of the measured degrees of agglutination, and the actually measured haptoglobin concentrations were calculated. A ratio near 1 indicated that the haptoglobin in the

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serum sample was of the Hp 2-2 phenotype, a smaller ratio, more particularly a ratio near 0.5 indicated that the haptoglobin in the serum sample was of the Hp 2-1 phenotype, and a very small ratio, namely near 0.12 indicated that the haptoglobin in the serum sample was of the Hp 1-1 phenotype.

From the above example, it can be concluded that, in view of the significant difference between the degrees of agglutination, at a same concentration, for the different haptoglobin phenotypes, the method according to the present invention is a very reliable and fast method for phenotyping haptoglobins.

Finally, it will be clear that many modifications can be applied to the above described methods and corresponding kits without leaving the scope of the present invention.

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### CLAIMS

1. A method for determining the phenotype of a haptoglobin in a biological fluid, characterized in that it comprises the steps of:
  - a) contacting the biological fluid with a haptoglobin binding partner which has at least two locations by which it can bind to haptoglobins so as to be able to agglutinate haptoglobins and/or to be agglutinated thereby to different degrees, depending on the haptoglobin phenotype;
  - b) measuring the degree of agglutination ; and
  - c) determining, based on the degree of agglutination, the phenotype of the haptoglobin in the biological fluid.
2. A method according to claim 1, characterized in that the locations by which said binding partner can bind to haptoglobins are formed by a peptide, a monoclonal or polyclonal antibody, an  $F_{ab}$ ,  $F_{ab'}$  or  $F(ab')_2$  fragment of an antibody, a lectin, a cell receptor, a molecular imprint of a haptoglobin, a bacterial antigen, and/or fragments thereof containing the respective haptoglobin binding site.
3. A method according to claim 2, characterized in that said locations are formed by a T4 antigen or a haptoglobin binding fragment thereof.
4. A method according to claim 1 or 2, characterized in that said locations or the entire haptoglobin binding partner is formed by an antibody which specifically binds an  $\alpha 2$  chain of a haptoglobin of phenotype Hp 2-1 or Hp 2-2 but not an  $\alpha 1$  chain.
5. A method according to claim 4, characterized in that said antibody specifically binds an epitope selected within the following amino acid sequence of an  $\alpha 2$  chain: ala val gly asp lys leu pro glu cys glu ala asp asp gly gln pro pro pro lys cys ile and comprising at least the following sequence: glu ala asp.

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6. A method according to any one of the claims 1 to 5 characterized in that said binding partner consists mainly of a molecule, possibly made by chemical binding of two or more molecules so as to provide said at least two haptoglobin binding locations, which molecule is able to agglutinate haptoglobins of different phenotypes to different degrees.

7. A method according to claim 6, characterised in that said binding locations are specific for different haptoglobin phenotypes in order to obtain said different degree of agglutination.

8. A method according to claim 6 or 7, characterised in that said molecule has a size such that said different degree of agglutination is obtained.

9. A method according to any one of the claims 1 to 5, characterized in that said binding partner comprises carrier particles.

10. A method according to claim 9, characterized in that said carrier particles comprise eucaryotic and/or procaryotic cells or a fraction thereof carrying receptors forming said locations to which haptoglobins can bind.

11. A method according to claim 10, characterized in that said cells are cells of *Streptococcus pyogenes* carrying in particular T4 antigens as haptoglobin receptors.

12. A method according to claim 10, characterized in that said cells are animal or human cells, in particular leukocytes, carrying CD receptors, in particular CD22 receptors.

13. A method according to claim 9, characterized in that said binding partner comprises carrier particles having said locations attached thereon, preferably by adsorption and/or covalent binding, said particles comprising in particular synthetic or mineral particles or natural cells or fragments thereof.

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14. A method according to claim 13, characterized in that, in case binding locations are specific, said carrier particles have a diameter of between 0.02 and 2  $\mu$ , and preferably, in case said binding locations are specific for different haptoglobin phenotypes, of between 0.1 and 0.8  $\mu$ , and, in case said binding locations are not specific for different haptoglobin phenotypes, of between 0.5  $\mu$  and 4  $\mu$ , and more preferably of between 1  $\mu$  and 3  $\mu$ .

15. A method according to claim 13 or 14, characterized in that the carrier particles have bacterial antigens, in particular T4 antigens, haptoglobin antibodies which are specific for the different haptoglobin phenotypes or not, and/or haptoglobin binding fragments of said antigens or antibodies adhered thereto to form said locations by which the binding partner can bind to haptoglobin.

16. A method according to any one of the claims 1 to 15, characterized in that, in addition to the degree of agglutination, the concentration of the haptoglobin in the biological fluid is also measured, and the phenotype of the haptoglobin in the biological fluid is determined on the basis of the concentration of the haptoglobin in the biological fluid and the degree of agglutination.

17. A method according to claim 16, characterized in that the relationship between the concentration of a reference haptoglobin in the biological fluid and the degree of agglutination is first established for at least one reference haptoglobin phenotype, in particular for haptoglobin phenotype Hp 2-1 and/or Hp 2-2, and preferably for haptoglobin phenotype Hp 2-2, and the phenotype of the haptoglobin in the biological fluid is determined by comparing the relationship between the measured concentration of the haptoglobin and the measured degree of agglutination with said established relationship between the

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concentration of the reference haptoglobin and the degree of agglutination obtained by said reference haptoglobin.

18. A method according to claim 17, characterized in that for determining the phenotype of the haptoglobin in the biological fluid, the measured degree of agglutination is correlated by means of said relationship established for the reference haptoglobin to a particular concentration of this reference haptoglobin, the ratio between this particular concentration and the measured haptoglobin concentration is calculated, and based on this ratio the phenotype of the haptoglobin is determined taking into account that, when taking haptoglobin Hp 2-2 as reference haptoglobin, the calculated ratio would be on average near 1; 0.5 and 0.125 respectively in case the haptoglobin in the biological fluid is of phenotype Hp 2-2, Hp 2-1 and Hp 1-1.

19. A method according to any one of the claims 1 to 18, characterized in that the degree of agglutination is measured nephelometrically and/or turbidimetrically.

20. A kit for determining the phenotype of a haptoglobin in a biological fluid, characterized in that it comprises:

- a) a haptoglobin binding partner which has at least two locations by which it can bind to haptoglobin so as to be able to agglutinate haptoglobins and/or to be agglutinated thereby to different degrees, depending on the haptoglobin phenotype; and
- b) at least one sample containing an haptoglobin of a predetermined phenotype.

21. A kit according to claim 20, characterized in that the haptoglobin in said sample is of the Hp 2-2 or Hp 2-1 phenotype, and preferably of the Hp 2-2 phenotype.

22. A kit according to claim 20 or 21, characterized in that it further comprises at least one reagent to measure the concentration of

haptoglobins, in particular a preparation of antibodies which bind to the different haptoglobin phenotypes.

23. Use of the method according to any one of the claims 1 to 19 or of the kit according to any one of the claims 20 to 22 for assessing atherosclerotic risk, wherein the determination of a Hp 2-2 haptoglobin phenotype is correlated with a higher atherosclerotic risk than the determination of a Hp 1-1 or 2-1 haptoglobin phenotype.

24. Use of the method according to any one of the claims 1 to 19 or of the kit according to any one of the claims 20 to 22 for estimating a patient's prognosis following viral infection, in particular a HIV infection, wherein the determination of a Hp 2-2 haptoglobin phenotype is correlated with a worsened prognosis than the determination of a Hp 1-1 or 2-1 haptoglobin phenotype.

25. Use of the method according to any one of the claims 1 to 19 or of the kit according to any one of the claims 20 to 22 for determining graft survival following transplantation, in particular liver transplantation, wherein the determination of a Hp 2-2 haptoglobin phenotype is correlated with a lower survival than the determination of a Hp 1-1 or 2-1 haptoglobin phenotype.

26. Use of the method according to any one of the claims 1 to 19 or of the kit according to any one of the claims 20 to 22 for improving the interpretation of laboratory parameters selected from the group comprising serum haptoglobin concentration, plasma lipids, in particular total lipids and LDL-cholesterol, plasma proteins, in particular ferritin, immunoglobulin A and ceruloplasmin, and other biochemical parameters such as serum iron, transferrin saturation, vitamin C and vitamin E, wherein the determination of a Hp 2-2 haptoglobin phenotype is correlated with other reference values than the determination of a Hp 1-1 or 2-1 haptoglobin phenotype.

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27. Use of a haptoglobin binding partner, which has at least two locations by which it can bind to haptoglobin so as to be able to agglutinate haptoglobins and/or to be agglutinated thereby to different degrees, depending on the haptoglobin phenotype, for determining the phenotype of a haptoglobin in a biological fluid, in particular in blood, plasma, serum, liquor, urine, cell extract or tissue extract.

28. Use according to claim 27, characterized in that the said locations on the haptoglobin binding partner are formed by a peptide, a monoclonal or polyclonal antibody, an  $F_{ab}$ ,  $F_{ab'}$  or  $F(ab')_2$  fragment of an antibody, a lectin, a cell receptor, a molecular imprint of a haptoglobin, a bacterial antigen, and/or fragments thereof containing the respective haptoglobin binding site.

29. Use according to claim 27 or 28, characterized in that the haptoglobin binding partner comprises bacterial cells, in particular cells of *Streptococcus pyogenes* carrying T4 antigens, or fractions thereof containing haptoglobin binding sites.

30. A haptoglobin binding partner for use in a method according to any one of the claims 1 to 19 and/or in the kit according to any one of the claims 20 to 22, characterized in that it is different from entire cells of *Streptococcus pyogenes* and has at least two locations by which it can bind to haptoglobin so as to be able to agglutinate haptoglobins and/or to be agglutinated thereby to different degrees, depending on the haptoglobin phenotype.

31. A haptoglobin binding partner according to claim 30, characterized in that it comprises carrier particles having said locations attached thereon, preferably by adsorption and/or covalent binding.

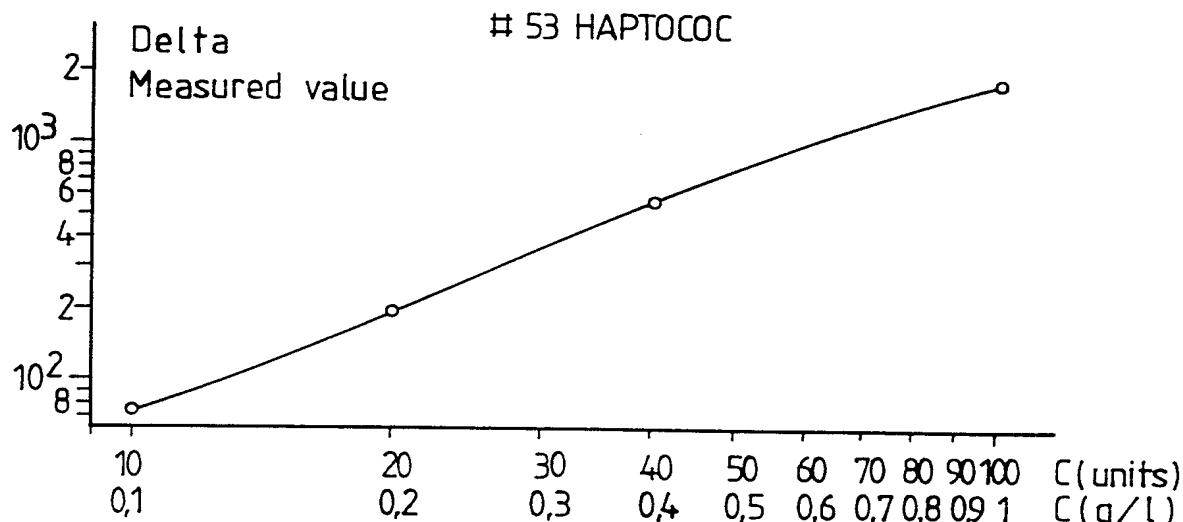
32. A haptoglobin binding partner according to claim 30 or 31, characterized in that it comprises an antibody or an antibody

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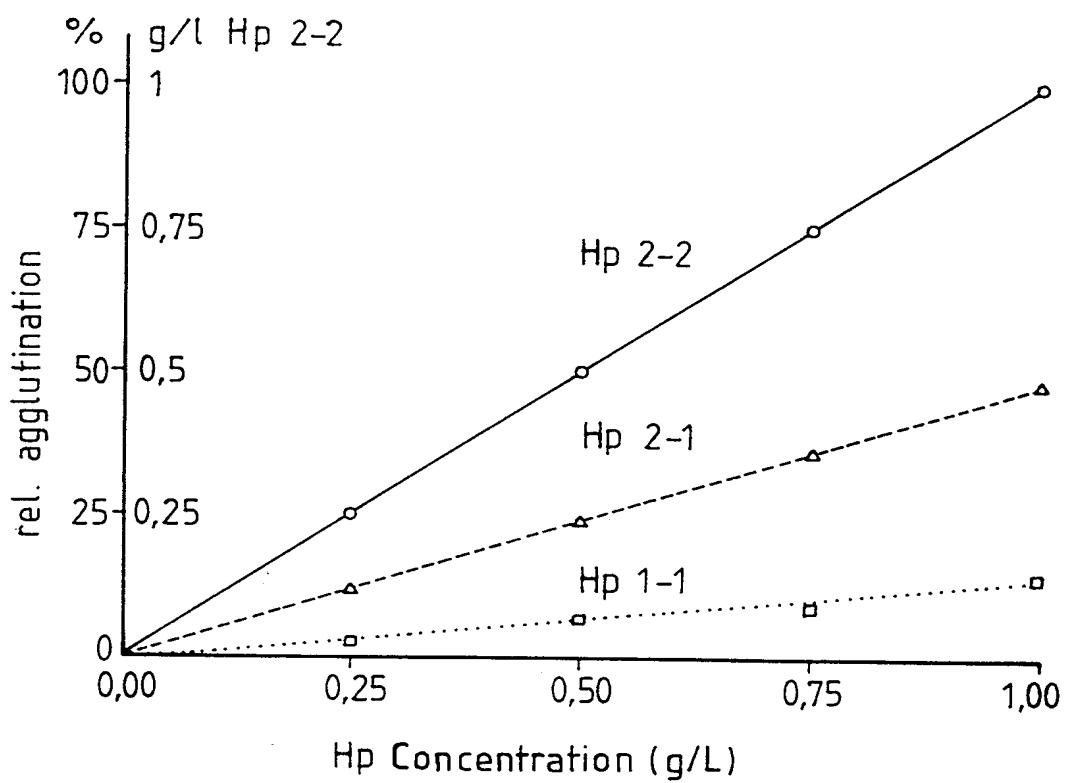
fragment which specifically binds an  $\alpha_2$  chain of a haptoglobin of phenotype Hp 2-1 or Hp 2-2 but not an  $\alpha_1$  chain.

33. A kit for determining the phenotype of a haptoglobin in a biological fluid, characterized in that it comprises a haptoglobin binding partner as defined in any one of the claims 30 to 32.

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**Fig.1**



**Fig.2**

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/BE 98/00023

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6 G01N33/68

According to International Patent Classification(IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 552 295 A (STANKER LARRY H ET AL) 3 September 1996 see the whole document ---	1,20,27
A	WO 90 08324 A (UNIV JOHNS HOPKINS) 26 July 1990 see the whole document ---	1,20,27 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

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European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

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Moreno, C

## INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE 98/00023

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CHEMICAL ABSTRACTS, vol. 124, no. 15, 9 April 1996 Columbus, Ohio, US; abstract no. 200157, L. WAGNER ET AL: "Haptoglobin phenotyping by newly developed monoclonal antibodies. Demonstration of haptoglobin uptake into peripheral blood neutrophils and monocytes." XP002071450 see abstract &amp; J. IMMUNOL., vol. 156, no. 5, 1996, pages 1989-1996, ----</p>	1,20,27
A	<p>CHEMICAL ABSTRACTS, vol. 85, no. 23, 6 December 1976 Columbus, Ohio, US; abstract no. 173693, J. M. RAMAKERS &amp; H. J. H. KREUTZER: "Turbidimetric determination of haptoglobin" XP002071451 see abstract &amp; J. CLIN. CHEM. CLIN. BIOCHEM., vol. 14, no. 8, 1976, pages 407-410, ----</p>	1,20,27
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